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Bone Marrow Stromal Cells as Targets for Gene Therapy of Hemophilia A

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ABSTRACT

Attempts to develop an *ex vivo* gene therapy strategy for hemophilia A, using either primary T cells or bone marrow (BM) stem/progenitor cells have been unsuccessful, due to the inability of these cell types to express coagulation factor VIII (FVIII). As an alternative, we evaluated the potential of BM-derived stromal cells which can be readily obtained and expanded *in vitro*. Human and murine BM stromal cells were transduced with an intron-based Moloney murine leukemia virus (MoMLV) retroviral vector expressing a B-domain-deleted human factor VIII cDNA (designated as MFG-FVIIIΔB). Transduction efficiencies were increased 10- to 15-fold by phosphate depletion and centrifugation, which obviated the need for selective enrichment of the transduced BM stromal cells. This resulted in high FVIII expression levels in transduced human (180 ± 4 ng FVIII/10^6 cells per 24 hr) and mouse (900 ± 130 ng FVIII/10^6 cells per 24 hr) BM stromal cells. Pseudotyping of the MFG-FVIIIΔB retroviral vectors with the gibbon ape leukemia virus envelope (GALV-*env*) resulted in significantly higher transduction efficiencies (100 ± 20%) and FVIII expression levels (390 ± 10 ng FVIII/10^6 cells per 24 hr) in transduced human BM stromal cells than with standard amphotropic vectors. This difference in transduction efficiency correlated with the higher titer of the GALV-*env* pseudotyped viral vectors and with the higher GALV receptor (GLVR-1) versus amphotropic receptor (GLVR-2) mRNA expression levels in human BM stromal cells. These findings demonstrate the potential of BM stromal cells for gene therapy in general and hemophilia A in particular.

OVERVIEW SUMMARY

This study demonstrates that bone marrow (BM) stromal cells may be used to achieve high levels of blood coagulation factor VIII expression *in vitro* after transduction with amphotropic Moloney murine leukemia virus (MoMLV) and gibbon ape leukemia virus envelope (GALV-*env*) pseudotyped retroviral vectors using optimized transduction protocols. Therefore, BM stroma may be useful as a target for gene therapy of hemophilia A.

INTRODUCTION

HEMOPHILIA A IS A CONGENITAL X-CHROMOSOME-LINKED coagulation disorder characterized by uncontrolled crippling hemorrhagic episodes that occurs in approximately 1/10,000 males (Sadler and Davie, 1987). Hemophilia A is due to a deficiency of coagulation factor VIII (FVIII), which accelerates the activation of factor X by activated factor IX in the presence of calcium and phospholipids. Ultimately, the coagulation cascade leads to the localized generation of thrombin and the conversion of fibrinogen to insoluble fibrin polymers, which in conjunction with platelet aggregation maintains hemostasis (for review, see Kaufman, 1991).

Hemophilia A is particularly suitable for gene therapy since expression of FVIII does not require precise metabolic regulation and since a slight increase in plasma FVIII levels can potentially convert severe (FVIII, 1–2 ng/ml) to moderate or mild hemophilia (FVIII, 2–60 ng/ml). Gene therapy for hemophilia A should provide constant, sustained synthesis within the patient, thereby obviating the risk of spontaneous bleeding, the need for repeated FVIII infusion (Thompson *et al.*, 1991; Morgan and Anderson, 1993; Lozier and Brinkhous, 1994) and the

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risk of viral infections associated with plasma-derived FVIII. Retroviral vector-mediated gene transfer offers the potential for long-term gene expression by virtue of its stable chromosomal integration and lack of viral gene expression. We and others have developed retroviral vectors for the transfer and expression of a B-domain deleted FVIII gene (Hoeben *et al.*, 1990, 1992, 1993; Israel and Kaufman, 1990; Lynch *et al.*, 1993; Chuah *et al.*, 1995; Dwarki *et al.*, 1995). The B-domain is not required for FVIII activity and is post-translationally cleaved off from the FVIII precursor (Toole *et al.*, 1986).

A major problem of FVIII retroviral vectors that has hampered its clinical applications for gene therapy is that both vector titer and FVIII protein production are 100- to 1,000-fold lower in comparison to vectors carrying other cDNAs (Hoeben *et al.*, 1990, 1992, 1993; Israel and Kaufman, 1990; Lynch *et al.*, 1993; Chuah *et al.*, 1995). A 1.2-kb inhibitory region has been identified by deletion analysis of the FVIII cDNA (Lynch *et al.*, 1993) that inhibited FVIII mRNA accumulation by inhibiting transcriptional elongation (Koeberl *et al.*, 1995), whereas conservative mutagenesis of the entire 1.2-kb inhibitory region did not restore viral titer and FVIII expression (Chuah *et al.*, 1995). However, inclusion of an intron upstream of the FVIII cDNA led to a significant increase in FVIII expression and retroviral titer (Chuah *et al.*, 1995; Dwarki *et al.*, 1995). In the present study, the intron-containing MFG-based FVIII retroviral vector (Danos and Mulligan, 1988; Dwarki *et al.*, 1995) was used because it was shown to express very high levels of FVIII in various cell lines as well as primary cells.

Attempts at achieving long-term human FVIII expression by *ex vivo* gene therapy approaches using a variety of primary cells have shown that access of the engineered cells to the bloodstream (such as by intrasplenic or intravenous injection) is a prerequisite to obtain detectable FVIII levels in the circulation (Hoeben *et al.*, 1993; Zatloukal *et al.*, 1994; Dwarki *et al.*, 1995). However, cells belonging to the lympho-hematopoietic lineage that can be stably transduced with retroviral vectors *ex vivo*, do not secrete detectable levels of FVIII protein (Hoeben *et al.*, 1992; Chuah *et al.*, 1995; Evans *et al.*, personal communication; Chuah and VandenDriessche, unpublished observations). These observations prompted us to identify new target cells for hemophilia A gene therapy.

Bone marrow (BM) stroma is a potentially attractive target for gene therapy of hemophilia A. BM stroma can relatively easily be obtained by BM aspiration, expanded *in vitro*, and transduced by retroviral vectors (Drize *et al.*, 1992; Nolta *et al.*, 1994; Li *et al.*, 1995). It is not known whether BM stromal cells can be engineered to express FVIII. Gene-marking studies have indicated that human or murine BM stromal cells engrafted in animal models can persist (Anklesaria *et al.*, 1987; Drize *et al.*, 1992; Li *et al.*, 1995; Prockop, 1997) and express the gene of interest at physiological levels for several months (Nolta *et al.*, 1994).

Prior to evaluating the potential of primary BM stromal cells for gene therapy of hemophilia in animal models, high retroviral transduction efficiency and FVIII expression needed to be established. Recently, it has been demonstrated that transduction efficiencies were increased in primary human T-cells transduced with Gibbon ape leukemia virus (GALV) *env* pseudotyped retroviral vectors as compared to amphotropic Moloney murine leukemia virus (MoMLV) vectors (Bunnell *et al.*, 1995). In addition, because these receptors are phosphate transporter molecules (Miller and Miller, 1994; Miller *et al.*, 1994; Kavanaugh *et al.*, 1994), their expression levels could be induced metabolically by phosphate starvation, resulting in higher transduction efficiencies (Bunnell *et al.*, 1995). The inclusion of a centrifugation step (Kotani *et al.*, 1994; Bunnell *et al.*, 1995) in combination with phosphate starvation further boosted transduction efficiency of primary T cells. On the basis of these observations, the transduction efficiency of primary human BM stromal cells was evaluated by using GALV-*env* pseudotyped MFG-FVIIIΔB versus amphotropic MoMLV in combination with an optimized transduction protocol involving phosphate starvation and centrifugation. This technique may not only increase the initial pool of transduced BM stromal cells, but also obviate the need for selection prior to reimplantation, possibly allowing transplantation of large quantities of retrovirally transduced cells for hemophilia A gene therapy.

MATERIALS AND METHODS

Cell lines and culture conditions

The murine fibroblast-like cell line NIH-3T3, the PG13 cell line (Miller *et al.*, 1991; provided by Dr. A.D. Miller, Fred Hutchinson Cancer Research Center, Seattle, WA), and its derivative PG13-F8 (clone #5), the Ψ-CRIP-derived amphotropic producer cell lines MFG-FVIIIΔB (clone # XF2) (Dwarki *et al.*, 1995; provided by Somatix, Inc., Alameda, CA), the GCsamF8EN producer clones (Chuah *et al.*, 1995) were cultured in Dulbecco's modified Eagle's medium (DMEM) with 2 m*M* L-Gln, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 10% heat-inactivated fetal bovine serum (FBS; designated as D10 medium) (Life Technologies, Merelbeke, Belgium). All cells, including the human and murine BM stromal cells, were grown at 37°C in an incubator with 95% humidity and 5% CO_2.

Isolation and in vitro *culture of primary human and murine BM stromal cells*

Primary human BM stromal cells were obtained by standard aspiration from freshly isolated BM from the iliac crest and/or sternum of healthy BM donors and collected in an equal volume of MyeloCult H5100 medium (StemCell Technologies, Vancouver, Canada) supplemented with 250 U/ml heparin. Erythrocytes were removed by sedimentation for 30 min at room temperature using Plasmasteril (Fresenius, Wilrijk, Belgium). The cells remaining in suspension were washed three times in phosphate-buffered saline (PBS) and seeded in 10-cm dishes at densities corresponding to 30×10^6 cells/15 ml of MyeloCult H5100 long-term culture medium supplemented with freshly prepared hydrocortisone (10^{-6} *M*, Sigma, Bornem, Belgium), 100 IU/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B (Life Technologies) (designated as HBM medium). BM stromal cells started to adhere 5–7 days after BM isolation, at which point 10 ml freshly prepared HBM medium was added. After expanding the BM stromal cells for an additional 7 days, the nonadherent suspension cells were decanted whereas the adherent stromal layer was trypsinized for transduction or further characterization.

The mouse BM stroma was isolated by sacrificing *bnx* mice (Harlan, Zeist, the Netherlands) and flushing the femur and tibia

with MyeloCult M5300 medium (StemCell Technologies). BM cells harvested either from one femur or two tibiae were cultured in a 10-cm petri dish containing 10 ml of MyeloCult M5300 long-term culture medium supplemented with freshly prepared hydrocortisone hemisuccinate (10^{-6} *M*, Sigma), 100 IU/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B (Life Technologies) (designated as MBM medium). Adherent mouse BM stromal cells were obtained by growing the cells for 3–7 days. After expanding the BM stromal cells for 7 days, the nonadherent suspension cells were decanted while the adherent stromal layer was trypsinized for transduction.

Vectors

The MFG-FVIIIΔB and the GCsamENF8 splicing vectors were described previously (Chuah *et al.*, 1995; Dwarki *et al.*, 1995) and were kindly provided by Somatix Corporation and Dr. R.A. Morgan (National Institutes of Health, Bethesda, MD), respectively. In these vectors, the B-domain-deleted FVIII gene was driven from the 5′ MoMLV long terminal repeat (LTR) and was cloned downstream of the MoMLV intron used to generate subgenomic *env* mRNA. In contrast to the GCsamF8EN vector, a Kozak consensus sequence for translational initiation was introduced in the MFG-FVIIIΔB vector and the 3′ untranslated region (UTR) of the FVIII gene was deleted. The MFG-FVIIIΔB vector lacked a *neo*^{R} selectable marker, whereas the GCsamF8EN vector expressed FVIII and the neomycin phosphotransferase II (NPTII) proteins from a single polycistronic transcript that was driven from the 5′ MoMLV LTR by virtue of the internal ribosome entry site (IRES).

*Generation and characterization of FVIII retroviral vectors pseudotyped with GALV-*env

Viral supernatant was first collected over 24 hr from a confluent plate of MFG-FVIIIΔB producer cells (clone #XF2) and filtered through a 0.45-μm filter to remove residual producer cells. To generate the MoMLV/GALV-*env* pseudotyped retroviral vectors, 8×10^6 PG13 cells were subjected to successive daily transductions with 5 ml of MFG-FVIIIΔB viral vector-containing conditioned medium in the presence of Polybrene (8 μg/ml, Sigma). The resulting producer cells were designated as PG13-F8 and individual clones were obtained by limiting dilution. FVIII production by each of the individual PG13-F8 clones was quantified using a functional chromogenic assay as described below. Clones that expressed the highest levels of FVIII were further screened for viral production by RNA dot blot analysis (see below) and subsequently subjected to Southern blot analysis as described previously (Sambrook *et al.*, 1989; Chuah *et al.*, 1995) to exclude the presence of rearranged proviral sequences. Briefly, genomic DNA was extracted with the high pure PCR template preparation kit (Boehringer, Mannheim, Germany), and 23 μg of DNA was restricted with *Sma* I or *Nhe* I. Hybridizations were performed by probing the Southern blot membrane with a FVIII-specific probe corresponding to a random primed 1,095-bp *Bgl* II–*Spe* I restriction fragment of plasmid pMT2LA. The membranes were washed stringently at 65°C in 2× SSC and 0.1% SDS for 30 min, followed by 0.5 × SSC and 0.2% SDS for an additional 30 min.

FVIII quantification

FVIII activity in the transduced stromal cells and the viral producer cell clones was quantified by measuring the FVIII-dependent generation of factor Xa from faactor X using a chromogenic assay (Coatest FVIII, Chromogenix, Molndal, Sweden), as described previously (Chuah *et al.*, 1995). Briefly, 24-hr conditioned culture medium was harvested in phenol-red free media to avoid colorimetric interference in the FVIII chromogenic assay. Human plasma purified FVIII (Octapharma, Langenfeld, Germany) of known activity was used as a FVIII standard and 1 unit was defined as 200 ng of FVIII/ml. The lowest level of detectable FVIII was 0.01–0.03 ng/ml and media containing heat-treated FBS did not yield any detectable FVIII activity.

Viral production and titration

Because MFG-FVIIIΔB did not contain a selectable marker, viral titer was determined by RNA dot blot analysis. Supernatant containing retroviral vector particles were obtained by seeding 4×10^6 producer MFG-FVIIIΔB and 30×10^6 producer PG13-F8 in 10 ml of D10 per 75-cm^2 flask, unless indicated otherwise. These cells were grown at 32°C and the supernatant was harvested after 24 hr. Supernatants were aliquoted and immediately frozen on dry ice prior to storing at −80°C until use. Vector titer in the culture medium was determined by RNA dot blot analysis as described previously (Yang *et al.*, 1995). Hybridizations were performed by probing the membrane with a FVIII-specific probe corresponding to a random-primed 1,095-bp *Bgl* II–*Spe* I restriction fragment of pMT2LA. The membranes were washed stringently at 65°C in 2× SSC for 30 min followed by 0.5× SSC for an additional 30 min. After background subtraction, signal intensities were quantified using a Phosphorimager (Molecular Dynamics, Sunnyvale, CA). Additional controls consisted of serially diluted viral vector supernatants with known functional titer based on vectors containing a *neo* gene (GCsamF8EN). Functional titers expressed as G418^R cfu/ml were determined by transduction of NIH-3T3 cells as described previously (Chuah *et al.*, 1994, 1995).

Immunohistochemical analysis of human BM stroma

Human BM stromal cells were seeded at a concentration of 10^4 cells per well in 0.4 ml of Iscove's modified Dulbecco's medium (IMDM) supplemented with freshly prepared hydrocortisone (10^{-6} *M*, Sigma), 10% heat-inactivated FBS, 2 m*M* L-Gln, 100 IU/ml penicillin, 100 μg/ml streptomycin, and 250 ng/ml amphotericin B (designated as I10 medium) in eight-chamber slides (Life Technologies) and incubated at 37°C. Medium was removed from chamber slides containing confluent cells followed by fixation with acetone at −20°C for 5 min. Fixed cells were washed twice in Tris saline buffer (TSB) pH 7.6 (100 m*M* Tris, 150 m*M* NaCl, 2 grams/liter Merthiolate, 10 ml/liter Triton X-100). After removing excess TSB, the cells were incubated for 30 min in TSB containing 0.3% H_2O_2 to consume potential endogenous peroxidase activity, and subsequently washed twice with TSB. Immunostaining was performed by first incubating the cells for 30 min in 100 μl of five-fold-diluted preimmune rabbit serum (Dako, Glostrup,

Denmark), after which supernatant was removed. For the fibroblast-specific immunostaining, the cells were incubated overnight with a monoclonal mouse antibody that reacted with human prolyl 4-hydroxylase (clone 5B5, 155 μg/ml Dako) diluted 1/50 to 1/100 in TSB (Hoyhtya *et al.*, 1984). For the endothelial cell-specific immunostaining, the cells were incubated with a monoclonal antibody (4H1D7) specific for von Willebrand factor (vWF) (diluted 1/300 from a 2.6 mg/ml stock) that was kindly provided by Dr. Marc Hoylaerts (KU Leuven, Belgium). The next day, cells were washed twice in TSB and incubated for 1 hr in 100 μl of diluted (1/400) biotinylated rabbit anti-mouse immunoglobulins (800 μg/ml, Dako). Subsequently, the cells were washed twice for 5 min in Tris pH 7.5 (500 m*M*) and incubated for 1 hr with avidin–biotin complex peroxidase (ABC-PO, Dako). After washing twice for 5 min in Tris, cells were stained with 240 ml of Tris containing 30 mg of diaminobenzidine (DAB) and 78 μl of H_2O_2 (30%). The reaction was terminated by washing the cells in TSB and counterstaining was performed using Harris' hematoxylin. After dehydrating the cells in gradually increasing concentrations of ethanol and xylol, slides were embedded in DPX resin (Prosan, Gent, Belgium).

Analysis of GLVR-1 *and* GLVR-2 *expression*

The relative *GLVR-1* and *GLVR-2* mRNA expression levels in the human BM stromal cells were determined by reverse transcriptase-polymerase chain reaction (RT-PCR). Total RNA was first purified using the chaotropic Trizol method followed by phenol-chloroform extraction and isopropanol precipitation (Chomczynski, 1993). The precipitated RNA was then redissolved in H_2O and spectrophotometrically quantified. The first-strand cDNA was synthesized starting from 1, 2.5, and 5 μg of purified total RNA using a Superscript II reverse transcriptase kit (Life Technologies). Two microliters of the cDNA was subjected to serial two-fold dilutions (ranging from 2- to 128-fold) prior to PCR amplification to ensure that detection of the PCR-amplified fragments fell within the linear range. PCR was performed with a Techne/Progene thermocycler using *Taq* polymerase and oligonucleotide pairs that discriminated specifically between GLVR-1 (5′-GCAGTTTTCTGTGCCCTTATCGTC-3′ and 5′-GGAGTTTATTTGGTTGCTGACGG-3′) and GLVR-2 (5′-TTCAGGAAGCAGAGTCCCCAGT-3′ and 5′-TGTCGATGTGGATTTTGTGCAG-3′) as has been corroborated previously (Lam *et al.*, 1996). As a control, β-actin-specific primers (5′-CATTGTGATGGACTCCGGAGACGG-3′ and 5′-CATCTCCTGCTCGAAGTCTAGAGC-3′) were added to the PCR reaction mixture. PCR reactions of the experimental samples and the standards were performed in the presence of the three primer sets (*i.e.*, GLVR-1, GLVR-2, and β-actin) simultaneously. PCR was performed by denaturation for 5 min at 94°C, followed by 26 cycles of 45 sec at 94°C, 1 min at 60°C, 2 min at 72°C, and a final extension for 7 min at 72°C. Standards corresponding to known concentrations of *GLVR-1* and *GLVR-2*-specific amplified fragments subjected to two-fold serial dilution (ranging from 0 to 4 pg) were used as controls for quantitative comparisons. These standards were generated by amplifying RT-PCR products from human T cells using the same PCR conditions as for the experimental samples, except that the cDNA was subjected to 35 rounds of amplification. These amplified fragments were then purified by Geneclean (Westburg, Leusden, The Netherlands) and the amounts were quantified spectrophotometrically. All amplified samples were separated by gel electrophoresis on the same 1.5% agarose gel and transferred to Hybond (Amersham, UK) membranes by Southern blotting as described previously (Sambrook *et al.*, 1989; Chuah *et al.*, 1994). Following prehybridization, the membranes were hybridized simultaneously with ^{32}P-end-labeled *GLVR-1*, *GLVR-2*-, and β-actin specific oligonucleotide pairs identical to the primers used for PCR. After overnight hybridization, the membranes were subjected to stringent washings and signal intensities were quantitated using a Phosphorimager apparatus.

Transduction of BM stromal cells

Human and murine BM stromal cells were seeded at a density of 10^5 cells/ml per well in a six-well plate containing 1 ml of 110 medium supplemented with freshly prepared hydrocortisone (10^{-6} *M*, Sigma) for human stroma or hydrocortisone-hemisuccinate (10^{-6} *M*, Sigma) for mouse stroma. The next day, the 110 medium was aspirated and BM stromal cells were washed once with 5 ml of PBS followed by transduction with the amphotropic MFG-FVIIIΔB vector (for murine and human stroma) or the GALV-*env* pseudotyped PG13-F8 vector (for human stroma) under standard or optimized conditions. Standard conditions involved overnight incubation of the stromal cells with vector-containing supernatant at 32°C by virtue of the increased stability of retroviral vectors at 32°C versus 37°C (Kotani *et al.*, 1994; Bunnell *et al.*, 1995; Kaptein *et al.*, 1997). Vector-containing supernatants were supplemented with 4 μg/ml of protamine sulphate and hydrocortisone (or hydrocortisone-hemisuccinate). Under optimized conditions, vector-containing supernatant with protamine sulfate and hydrocortisone (or hydrocortisone-hemisuccinate) was added to the cells followed by a centrifugation step at 32°C for 1 hr at 1,400 × *g* directly on six-well plates and on overnight incubation at 32°C. Cells were returned to 37°C the next day. For some transductions, a phosphate starvation step was included that involved a 9- to 10-hr incubation of the stromal cells in 110 medium containing IMEM without phosphate. A total of four rounds of transductions were performed successively under the same conditions over the next 4 days followed by two washings with PBS.

Analysis of transduction efficiency by quantitative PCR and Southern blot

High-molecular-weight genomic DNA was isolated from the transduced BM stromal cells using the high pure PCR template preparation kit (Boehringer, Mannheim, Germany). To determine transduction efficiency, PCR was performed with a Techne/Progene thermocycler using *Taq* polymerase and oligonucleotide pairs specific for the FVIII cDNA in the MFG-FVIIIΔB or GCsamF8EN retroviral vectors (5′-GAGCTCTCCACCTGCTTCTTTCTG-3′ and 5′-CCCTTCTCTACATACTAGTAGGGC-3′) yielding a specific 594-bp PCR product. For normalization, β-actin-specific primers (5′-CATTGTGATGGACTCCGGAGACGG-3′ and 5′-CATCTCCTGCTCGAAGTCTAGAGC-3′) were added to the PCR reaction mixture yielding a 232-bp β-actin-specific PCR product. A producer clone transduced with GCsamF8EN and containing six integrated proviral copies was used as a control. PCR was performed by denaturation for 8 min at 95°C, followed by 28 cycles of 1 min

at 95°C, 1 min at 59°C, 2 min at 72°C, and a final extension for 5 min at 72°C. Amplified samples were separated by gel electrophoresis on 1.5% agarose gels and transferred to Hybond (Amersham) membranes by Southern blotting as described previously (Sambrook *et al.*, 1989; Chuah *et al.*, 1994). Following prehybridization, the membranes were hybridized overnight to ^{32}P-end-labeled FVIII and β-actin-specific oligonucleotides that were labeled using polynucleotide kinase as described previously (Sambrook *et al.*, 1989). Membranes were washed under stringent conditions (65°C in 2× SSC for 30 min followed by 0.5× SSC for an additional 30 min) and quantitation was performed using a Phosphorimager. The PCR was repeated three times and a representative experiment is shown in Fig. 5 (below).

Statistical analysis

The results are represented as mean ± SD of triplicate experiments. The significance of the difference was determined using Student's *t*-test for unpaired values.

RESULTS

Characterization of human BM stromal cells

The kinetics of the development of an adherent BM stromal cell layer did not vary significantly among different BM donors. During the first 5 days, most BM cells remained in suspension except for the presence of adherent macrophage-like cells. Cells with a spindle-like morphology started to adhere between days 6 and 10 and continued to proliferate to generate an adherent layer (Fig. 1A). More than 95% of the adherent stromal cells exhibited the distinctive spindle-like morphology that was retained, even after long-term culture over several months. Immunostaining revealed that the majority of the stromal cells were fibroblastic (84 ± 8.0%) (Fig. 1B), whereas vWF-positive endothelial cells were absent. Additional controls without preimmune serum, primary, or secondary antibodies and NIH-3T3 fibroblasts yielded no staining whereas human umbilical vein endothelial cells (HUVEC) stained positively for vWF, as expected.

Comparison of GLVR-1 *and* GLVR-2 *expression in human BM stromal cells*

To correlate the efficiency of transduction of human BM stromal cells by amphotropic MoMLV or GALV-*env* pseudotyped retroviral vectors with the relative abundance of GLVR-1 (GALV-*env*) and GLVR-2 (amphotropic) receptor on the target cells (Lam *et al.*, 1996), their relative expression levels were determined by quantitative RT-PCR/Southern blot analysis, using GLVR-1- and GLVR-2-specific primer-pairs that amplified a nonhomologous region of 402 and 306 bp, respectively (Fig.

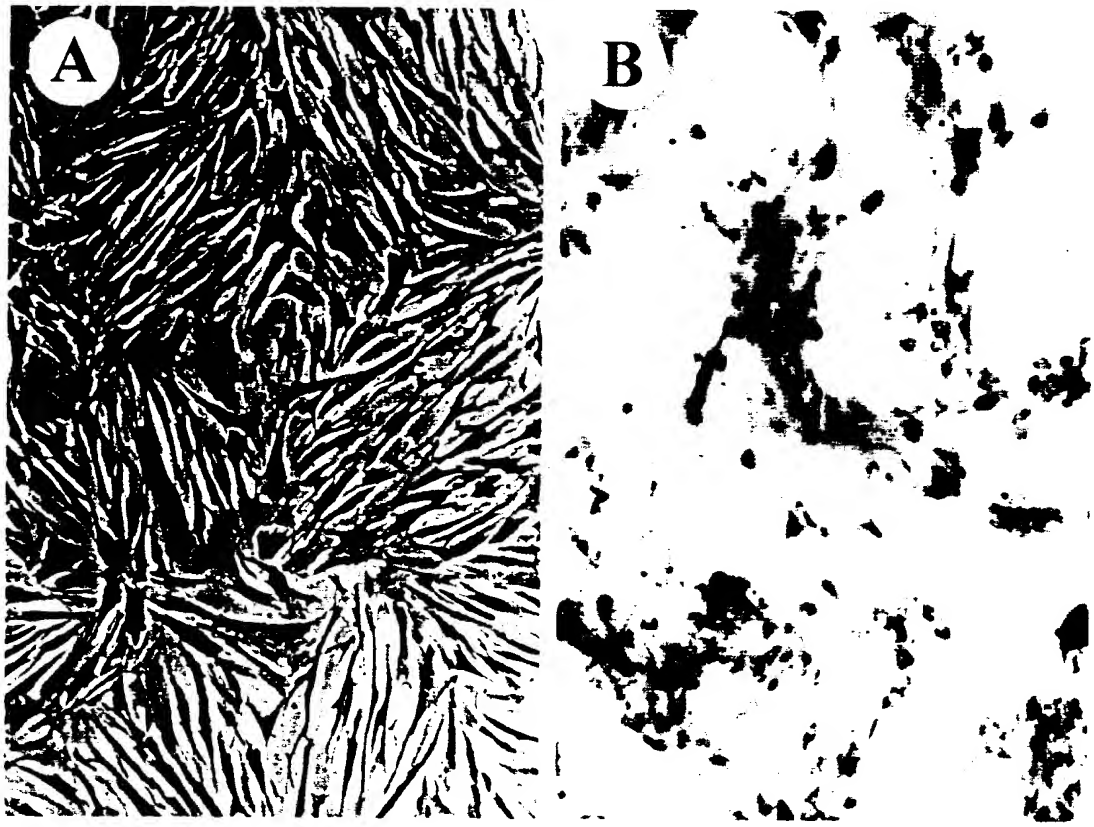
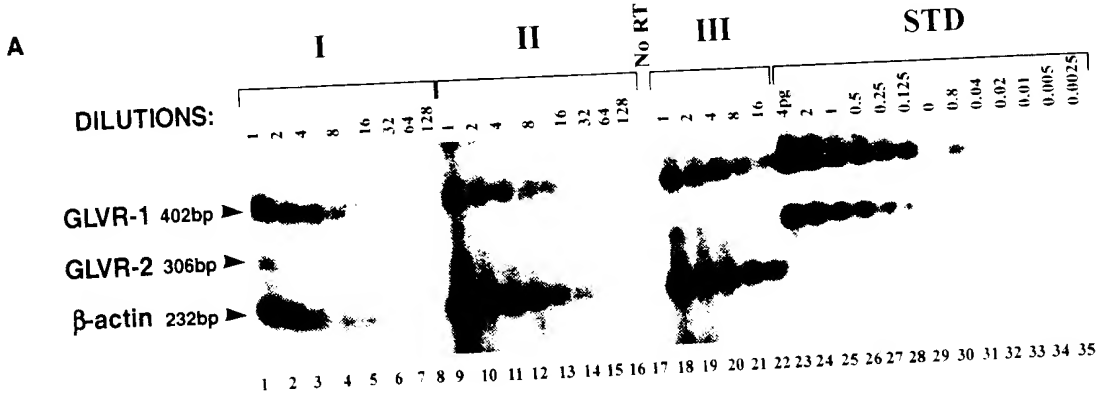


FIG. 1. Development of an adherent stromal layer after long-term BM culture and immunohistochemical staining. A confluent monolayer of subcultured cells at the time of transduction is shown (A). Human BM stromal cells were stained for human prolyl 4-hydroxylase as a fibroblast-specific marker (B).

2A) (Lam *et al.*, 1996). A linear standard curve (correlation coefficient, $r^2 = 0.98$) was obtained for known amounts of *GLVR-1* and *GLVR-2* cDNA fragments ranging between 0 to 0.125 pg (Fig. 2A, lanes 28–35). The PCR signal for the *GLVR-1* and *GLVR-2* cDNA standards was not linear anymore between 0.250 pg and 4 pg (lanes 23–27). Detection of *GLVR-1*- and *GLVR-2*-specific PCR-amplified fragments from the BM stromal cells fell within the linear range of the assay for the RT-PCR reaction products obtained from 1 μg of RNA (lanes 1–8) and 2.5 μg of RNA (lanes 9–16) ($r^2 = 0.97$–0.99). No PCR-amplified product could be detected in the negative controls that did not contain RT (lane 17), excluding amplification from potentially contaminating genomic DNA. As expected, no PCR product was observed in the absence of *GLVR-1* and *GLVR-2* cDNA templates (lane 29). The β-actin signal was only detectable in the experimental samples but not in the standards as expected, because the standards corresponded to purified GLVR-1 and GLVR-2 cDNA fragments (lanes 23–35).

Comparative analysis of GLVR-1 and GLVR-2 expression in human BM stromal cells determined from the amounts of *GLVR-1*- and *GLVR-2*-specific cDNA fragments at different dilutions indicated that GLVR-1 expression was six-fold higher than GLVR-2. In control experiments with human $CD4^+$ T lymphocytes, *GLVR-1* was expressed at high levels whereas only very low levels of *GLVR-2*-specific PCR fragments could be detected (data not shown), in accordance with previous observations (Lam *et al.*, 1996).



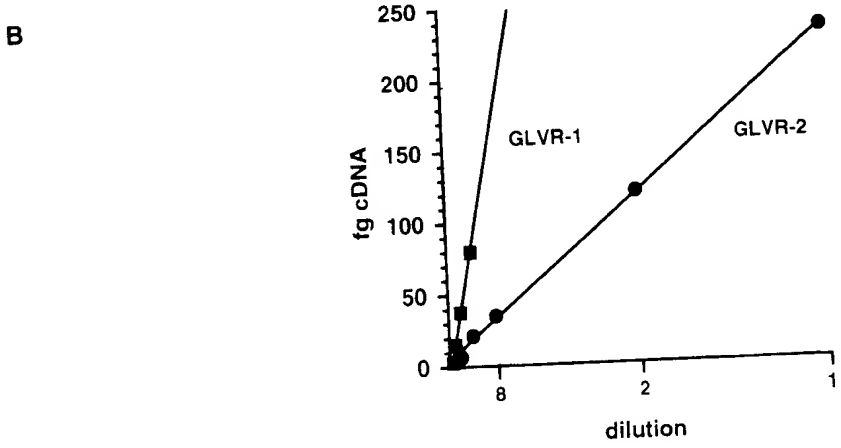


FIG. 2. Determination of *GLVR-1* versus *GLVR-2* mRNA expression by quantitative RT-PCR. Purified total RNA (1, 2.5, and 5 μg) from adherent human BM stromal cells was reverse transcribed. Two microliters of the reaction mixture containing the cDNA obtained form either 1 μg of RNA (I) (lanes 1–8), 2.5 μg of RNA (II) (lanes 9–16), or 5 μg RNA (III) (lanes 18–22) was serially diluted and subjected to PCR with *GLVR-1*- and *GLVR-2*-specific primers as described in Materials and Methods. Standards (STD) corresponding to known concentrations of *GLVR-1*- and *GLVR-2*-specific amplified fragments subjected to two-fold serial dilution (ranging from 0 to 4 pg, lanes 23–35) were used as controls for quantitative comparisons. Negative controls included samples without RT (lane 17) or without template (lane 29). Amplified samples were separated by gel electrophoresis on 1.5% agarose gels and subjected to Southern analysis with GLVR-1- and GLVR-2-specific oligonucleotides. Quantification was performed using a Phosporimager (Molecular Dynamics, Sunnyvale, CA) after background subtraction (B) using experimental samples that fell within the linear range of the assay corresponding to (lanes 13–16) (A) for GLVR-1 (cDNA obtained from 2.5 μg of RNA was diluted 16- to 128-fold) and (lanes 9–14) (A) for GLVR-2 (cDNA obtained from 2.5 μg of RNA was diluted 1- to 32-fold).

*Generation of the GALV-*env *pseudotyped PG13-F8 retroviral vector and comparison with the amphotropic MFGFVIIIΔB vector*

The FVIII retroviral vector used in the present study was derived from the MFGFVIIIΔB splicing vector rather than the GCsamF8EN splicing vector that we generated previously (Chuah *et al.*, 1995), because NIH-3T3 fibroblasts expressed seven- to 8-fold more FVIII when transduced with MFGFVIIIΔB than with GCsamF8EN. Similarly, FVIII expression was 10-fold higher in the cognate viral producer cell clones. In view of the higher GLVR-1 (GALV-*env*) versus GLVR-2 (amphotropic) receptor expression levels in human BM stromal cells (Fig. 2), GALV-*env* pseudotyped FVIII-retroviral vectors were generated (designated as PG13-F8) in an attempt to obtain high transduction efficiencies. Six out of the 23 PG13-F8 clones that were screened, expressed functional FVIII ranging from 5 to 200 ng FVIII/10^6 cells per 24 hr (the highest producer clone was designated as PG13-F8 clone #5). Southern blot analysis of high producer PG13-F8 clones revealed the presence of nonrearranged, intact FVIII-proviral sequences (data not shown). Using GCsamF8EN (Chuah *et al.*, 1995) for calibration with a functional titer of 5×10^5 $G418^R$ cfu/ml, RNA dot blot analysis yielded an equivalent average functional titer for MFG-FVIIIΔB of $6 \pm 3 \times 10^4$ $G418^R$ cfu/ml based on 23 independent batches of viral supernatant. The titer of the PG13-F8 clone #5 was equivalent to $(1.8 \pm 0.6) \times 10^5$ cfu/ml based on 17 independent batches of viral supernatant. This titer is consistent with previous reports using PG13-derived GALV-*env* pseudotyped retroviral vectors (Miller *et al.*, 1991; Bunnell *et al.*, 1995). When cells were seeded at similar densities, both the PG13-F8 pseudotyped producer cell clone #5 and the MFG-FVIIIΔB clone #XF2 exhibited similar titers, but because PG13-F8 clone #5 could be grown at higher cell densities significantly higher ($p < 0.001$) viral titers could consistently be achieved with the PG13-F8 producer clone #5 than with the best MFG-FVIIIΔB producer clone (Fig. 3).

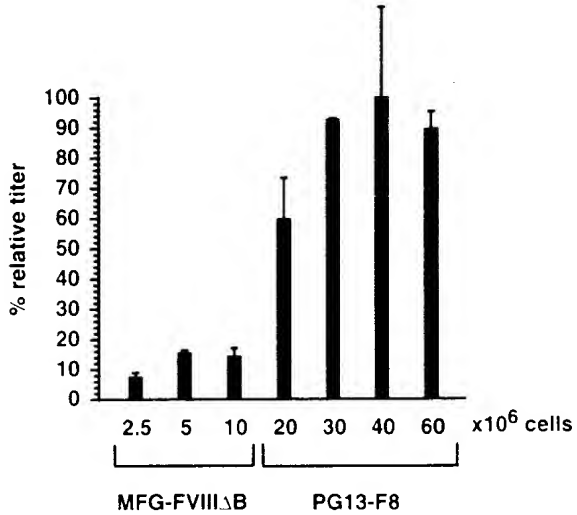


FIG. 3. Titration of FVIII-retroviral vectors pseudotyped with GALV-*env* (PG13-F8). Relative viral titer of the MFG-FVIIIΔB and PG13-F8 producer cell clones was determined as a function of cell number by RNA dot blot analysis of polyethylene glycol (PEG)-precipitated viral vector particles using a FVIII-specific probe as described in the Materials and Methods. Quantification was performed using a Phosphorimager after background subtraction.

Comparison of FVIII expression and transduction efficiency in transduced BM stromal cells

FVIII production was three- to four-fold higher ($p < 0.001$) in human BM stromal cells transduced under standard conditions with GALV-*env* pseudotyped PG13-F8 vector than with the amphotropic MFG-FVIIIΔB vector (Fig. 4A). Under optimized conditions, which involved transduction by centrifugation in combination with phosphate starvation, FVIII expression was significantly increased six-fold ($p < 0.001$) with MFG-FVIIIΔB and three- to four-fold with PG13-F8 ($p < 0.001$) over the standard method. The centrifugation step alone (without phosphate starvation) led to a significant three- to four-fold ($p < 0.001$) enhancement of FVIII expression with either MFG-FVIIIΔB or PG13-F8. In contrast, a relatively moderate increase in FVIII expression level was observed after phosphate starvation with either vector ($p < 0.05$). The development of the GALV-*env* pseudotyped PG13-F8 retroviral vector in combination with the use of an optimized transduction protocol has led to a significant ($p < 0.001$) overall 12-fold increase in FVIII expression levels in transduced human BM stromal cells as compared to transductions performed under standard conditions with the amphotropic MFG-FVIIIΔB vector. These conclusions were based on three independent experiments and the results of one representative experiment are depicted.

The increase in FVIII expression using PG13-F8 vectors correlated with the six-fold higher GLVR-1 versus GLVR-2 expression levels and with the seven-fold higher titer of PG13-F8. To rule out that this titer difference would in itself be sufficient to account for the differences in FVIII expression levels, transductions of human BM stromal cells were performed under optimized conditions after normalizing the multiplicities of infection (moi) of the GALV-*env* pseudotyped PG13-F8 and amphotropic MFG-FVIIIΔB vectors. Even under these conditions, FVIII expression levels were significantly higher (1.5- to 2-fold; *t*-test, $p < 0.001$) (Fig. 4B) in the human BM stromal cells transduced with PG13-F8 than with the amphotropic MFG-FVIIIΔB vector, although the difference was less, as expected, than in the nonnormalized experiments when relatively higher titers of PG13-F8 were used (Fig. 4A).

In murine BM stromal cells, the optimized transduction protocol with the MFG-FVIIIΔB vector also yielded significantly higher FVIII expression levels ($p < 0.001$) as compared to the standard protocol (Fig. 4C). However, mouse cells are refractory to transductions with GALV-*env* pseudotyped retroviral vectors (O'Hara *et al.*, 1990). The FVIII expression levels in mouse BM stromal cells transduced with the MFG-FVIIIΔB vector were consistently higher than in human BM stroma transduced with the same vector, possibly due to the higher proliferative capacity of the mouse BM stroma at the inception of transduction resulting in a significant seven- to nine-fold increase ($p < 0.001$) in transduction efficiencies compared to human BM stroma.

Human BM stroma was transduced two- to three-fold ($p <$

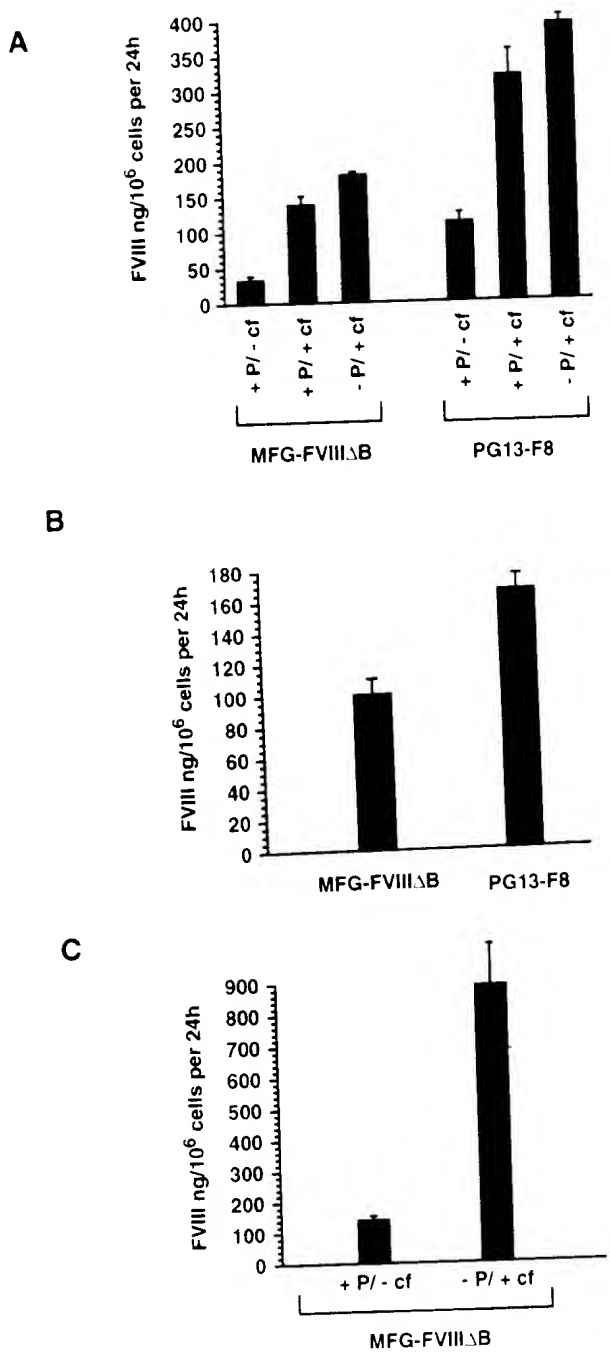


FIG. 4. FVIII production in transduced BM stromal cells. Human (A, B) or mouse (C) BM stromal cells were subjected to four successive rounds of transductions with either MFG-FVIIIΔB (A,B,C) or PG13-F8 (A,B). The titer and the moi of PG13-F8 was either seven-fold higher than MFG-FVIIIΔB (A) or normalized (B). Transductions were performed at 32°C with (+) or without (−) centrifugation (cf) or phosphate (P). FVIII production was determined with a functional chromogenic assay.

0.05) more efficiently with the GALV-*env* pseudotyped PG13-F8 retroviral vector-containing supernatant than with the amphotropic MFG-FVIIIΔB vector in standard and optimized conditions (Fig. 5A,B). The optimized transduction method led to a significant ($p < 0.005$) 10- to 15-fold increase in transduction efficiency compared to the standard method (Fig. 5A,B). The contribution of the centrifugation during the transduction was more important to the overall increase in transduction efficiency than the phosphate starvation since a significant 8- to 10-fold increase ($p < 0.05$) in transduction efficiency was observed when BM stromal cells were subjected to centrifugation during the transductions than when the centrifugation step was omitted (Fig. 5A,B). The differences in transduction efficiency correlated with the differences in FVIII expression levels in the transduced BM stromal cells (Fig. 5C).

In conclusion, the development of the GALV-*env* pseudotyped PG13-F8 retroviral vector in combination with the use of an optimized transduction protocol has led to an overall 20- to 30-fold increase in transduction efficiency in transduced human BM stromal cells as compared to transductions performed under standard conditions with amphotropic vectors. Human BM stroma transduced with PG13-F8 under optimized conditions contained an average of one to four integrated FVIII-proviral copies/cell, by calibrating against retroviral producer clone with known FVIII proviral copy number.

DISCUSSION

In the present study, we have demonstrated that human or mouse BM stromal cells can be exploited as an alternative BM-derived target cell for hemophilia A gene therapy because they could express relatively high levels of human FVIII (400–900 ng/10^6 cells per 24 hr) when transduced with FVIII retroviral vectors. The high levels of FVIII could be attributed to the use of an intron-based vector, the development of an optimized transduction protocol, and the generation of a GALV-*env* pseudotyped FVIII retroviral vector. Hence, a large number of FVIII-expressing primary BM stromal cells could be obtained while obviating the need to enrich for transduced cells by selection and without inducing stromal cell proliferation by supplementing high doses of exogenous purified growth factors. These improvements shorten the *in vitro* culture period of the BM stromal cells that are thus more likely to retain their original properties. Furthermore, because selective enrichment of transduced cells was not needed, it became undesirable to include a *neo*-selectable marker in the vector, especially since expression of the *neo* gene could trigger an immune rejection of the engineered cells.

The optimized transduction protocol involving centrifugation and phosphate starvation contributed to an increase in transduction efficiency of BM stromal cells both with the MFG-FVIIIΔB vector and its GALV-*env* pseudotyped counterpart, and concomitantly led to a significant increase in FVIII expression levels. The effect of the centrifugation was more pronounced than the effect of phosphate starvation. The mechanism by which centrifugation increased transduction efficiency is unclear but possibly involved perturbation of the cellular membrane, facilitation of the attachment of viral particle aggregates onto the cells, and/or increase in their local concen-

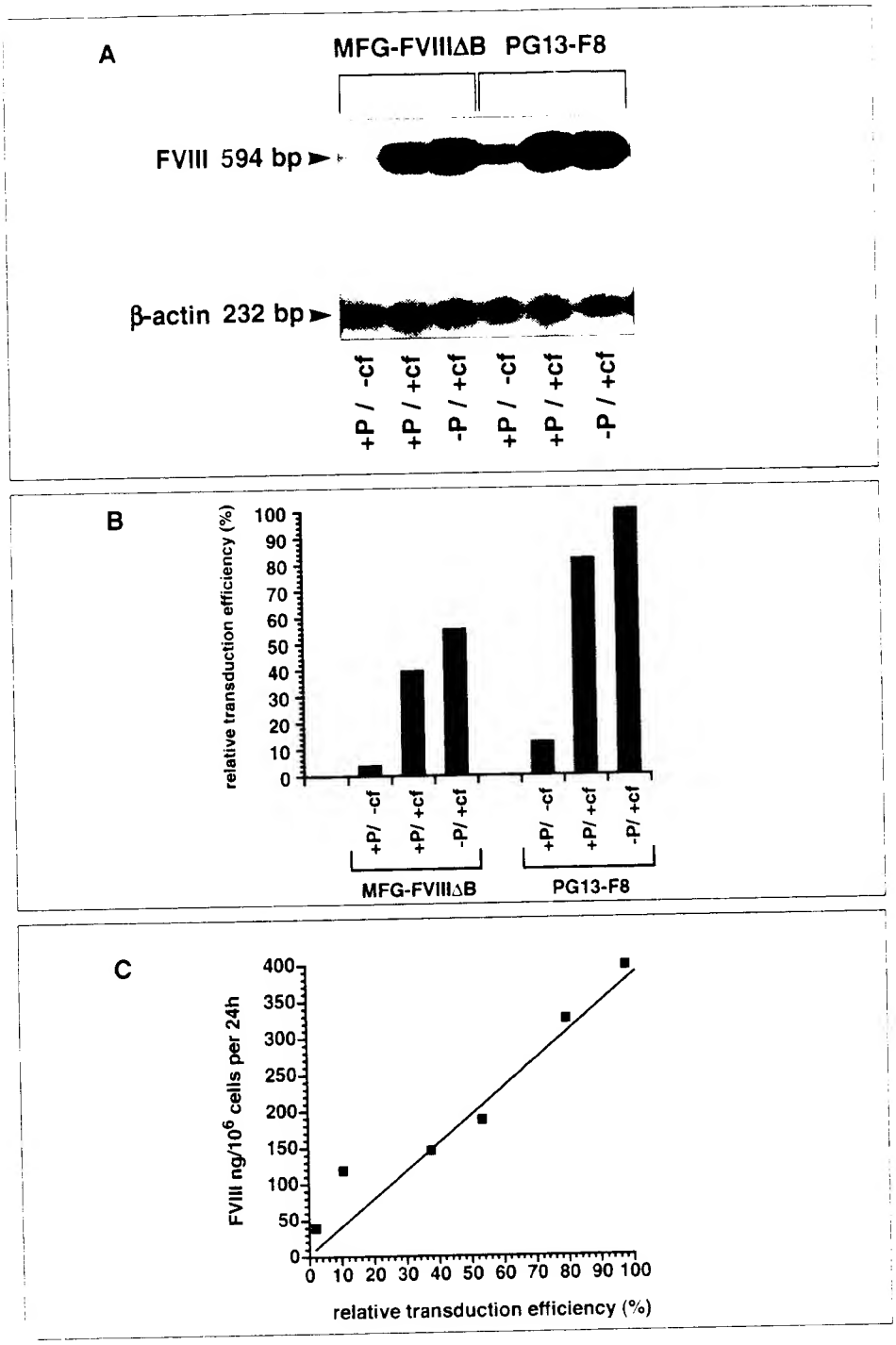


FIG. 5. Analysis of transduction efficiency by quantitative PCR/Southern blot analysis. Genomic DNA of the transduced human BM stromal cells was subjected to PCR/Southern blot analysis using primers specific for the FVIII-retroviral vector and β-actin-specific primers for normalization. Bands corresponding to the amplified FVIII or β-actin-specific fragments were indicated by arrows (A). All transduction quantifications of FVIII-specific PCR products, were always performed in the linear range of the assay (correlation coefficient, $r^2 = 0.96$) using serially diluted standards for verification. The intensities of the PCR-amplified fragments relative to the maximum transduction efficiency (*i.e.*, PG13-F8-transduced after centrifugation and phosphate starvation) were quantified with a Phosphorimager after background subtraction and β-actin normalization yielding the relative transduction efficiencies (%) (B). Transductions wee performed with either MFG-FVIIIΔB or PG13-F8 at 32°C with (+) or without (−) centrifugation (cf) or phosphate (P) as indicated (A,B). The FVIII expression was expressed in function of the relative transduction efficiencies of the human BM stromal cells transduced under all conditions tested (C).

tration. Our results were consistent with a recent study indicating that the NIH-3T3 cell line could be transduced more efficiently by incorporating a centrifugation step during the transduction (Kotani *et al.*, 1994). The phosphate starvation most likely resulted in a metabolic induction of receptor expression levels as was shown previously (Kavanaugh *et al.*, 1994) in rat fibroblasts. Furthermore, a combination of phosphate starvation and centrifugation also augmented transduction efficiency of amphotropic and GALV-*env* pseudotyped retroviral vectors in $CD4^+$ T lymphocytes (Bunnell *et al.*, 1995). However, in this case the effect of the phosphate starvation was much more pronounced, probably because of cell-type-dependent differences in induction of viral receptor expression.

Transduction of human BM stromal cells with the GALV-*env* pseudotyped retroviral vector was more efficient and consequently led to higher FVIII expression levels than with the amphotropic MFG-FVIIIΔB vector. This higher transduction efficiency correlated with higher levels of GLVR-1 than GLVR-2 expression in human BM stromal cells. Furthermore, increasing GLVR-1 expression by phosphate starvation resulted in increased transduction efficiency (and FVIII expression). These findings further support the notion that receptor concentration is rate limiting for transduction. The higher titer of the GALV-*env* pseudotyped PG13-F8 viral supernatants further boosted the transduction efficiency and contributed to the higher FVIII expression levels. Our results corroborated the notion that envelope switching by pseudotyping could influence transduction efficiency primarily as a consequence of differential receptor expression on the target cells as was shown recently in primary T lymphocytes (Bunnell *et al.*, 1995; Lam *et al.*, 1996; Porter *et al.*, 1996), primary BM stem/progenitor cells (Orlic *et al.*, 1996; Porter *et al.*, 1996), chronic myelogenous leukemia (CML) and Epstein–Barr virus (EBV)-immortalized B cell lines (Bauer *et al.*, 1995), and primary melanoma cultures (Porter *et al.*, 1996).

The human BM stromal cell population appeared to consist predominantly of fibroblastic cells whereas no vWF-positive endothelial-like cells could be detected in accordance with previous observations (Bentley and Foidart, 1980; Bentley and Tralka 1982), although other reports indicated that endothelial-like cells could be isolated from long-term BM cultures (Zuckerman and Wicha, 1983; Fei *et al.*, 1990; Hauser *et al.*, 1995). These differences may be due to culture conditions and/or kinetic differences of cell growth and differentiation. The spindle-shaped fibroblastic morphology, the growth kinetics, and the clonogenic appearance of the BM stromal cells are indicative of pluripotent mesenchymal (stromal) stem/precursor cells (Lazarus *et al.*, 1995; Prockop, 1997). At present, we cannot rule out the presence of other cell types at low densities such as macrophages, adipocytes, or smooth muscle cells. Although macrophage-like cells appeared to be present in the cultures initially, they were progressively eliminated by sequential trypsinizations and cell expansion, because terminally differentiated macrophages do not proliferate.

Previously, it has been shown that human FVIII could be expressed by engineering other cell types distinct from BM stromal cells. Transduction of primary human skin fibroblasts with retroviral vectors containing the B-domain-deleted FVIII cDNA led to relatively low levels of FVIII (3–39 ng/10^6 cells per 24 hr) (Hoeben *et al.*, 1990, 1993; Israel and Kaufman, 1990; Lynch *et al.*, 1993). Previously, we transduced a variety of cell types of epithelial, myoblastic, or endothelial origin with the GCsamF8EN splicing vector (Chuah *et al.*, 1995), but expression levels were again lower as compared to the levels obtained in BM stromal cells transduced with MFG-FVIIIΔB. The lower FVIII expression levels in these transduced cell types could be attributed at least partly to the type of retroviral vector used because FVIII expression levels in primary human skin fibroblasts, myoblasts and endothelial cells transduced with the MFG-based FVIII retroviral vector (Dwarki *et al.*, 1995; Krall *et al.*, 1996) were at least as high as the levels obtained in MFG-FVIIIΔB-transduced BM stromal cells. Human FVIII expression in primary mouse fibroblasts and myoblasts transfected by receptor-mediated FVIII gene delivery with polylysine-modified transferrin-adenoviral conjugates (Zatloukal *et al.*, 1994) was comparable to the levels obtained in MFG-FVIIIΔB-transduced BM stromal cells but expression was transient. In contrast, high levels of FVIII expression could be sustained for up to at least 6 months of continuous *in vitro* culture of the transduced BM stromal cells.

The rate of FVIII production by the transduced BM cells (K_0) to achieve a given steady-state concentration of FVIII protein in the plasma (C_{SS}) can be calculated based on the formula $K_0 = 24 \times 0.693\ V_d C_{SS}/T_{1/2}$ (Treco *et al.*, 1995), where $T_{1/2}$ is the plasma half-life of FVIII (12 hr) and V_d is the body distribution volume of FVIII (3,000 ml). To achieve FVIII plasma levels of 10 ng/ml (corresponding to mild hemophilia), an *in vivo* FVIII production of 42 μg/24 hr would be required. Clinical application for hemophilia A using BM stroma would thus require injection of at least 10^8 autologous BM stromal cells to convert a severe to a mild hemophiliac based on an *in vitro* production of 400 ng/10^6 cells per 24 hr. To correct the bleeding phenotype completely, an infusion of at least 8×10^8 BM stromal cells would be needed. The actual FVIII production *in vivo* will also depend on the engraftment efficiency and the promoter strength *in vivo*. Previous studies indicate that the MoMLV LTR, which was also used to drive FVIII expression in this study, is not shut down in human BM stromal cells *in vivo* (Nolta *et al.*, 1994). At least 10^{10} BM stromal cells could be enriched from 1 liter of total BM, which could routinely be obtained from a single BM isolation without any adverse side effects to the donor. Collection of 1 liter of autologous BM from a hemophiliac could be accomplished safely by supplying sufficient FVIII protein prophylactically, during and after the procedure to prevent bleeding. This should not be cause for concern, because even excessive bleeding in hemophiliacs suffering major trauma can be prevented by factor VIII protein infusions.

The ability of transplanted BM stromal cells to home and stably engraft into the marrow cavity continues to be the subject of controversy. Several independent investigators have found that transplanted stromal cells that could be distinguished from the recipient stroma by a genetic marker or a retroviral tag, did engraft *in vivo* (Keating *et al.*, 1982; Anklesaria *et al.*, 1987; Drize *et al.*, 1992; Ishida *et al.*, 1994; Li *et al.*, 1995; Prockop, 1997), even in the absence of myeloablation (Quesenberry *et al.*, 1994). *Ex vivo* expansion and subsequent infusion of human BM-derived stromal cells (Lazarus *et al.*, 1995) in phase I clinical trials was not associated with any adverse effects. Retrovirally marked BM stromal cells can be transplanted efficiently in mice and persisted long term *in vivo*.

even after transplantation in secondary recipients, suggesting that stromal precursor cells may have been transduced (Drize *et al.*, 1992). Ectopic transplantation of donor-derived stromal cells can replace the host stromal cells in host femurs, suggesting that BM stromal cells mobilized and migrated from the engrafted marrow to the host BM (Ishida *et al.*, 1994). These data were consistent with recent observations showing that human BM stromal cells transduced with a retroviral vector encoding human interleukin-3 (IL-3) gene could express physiological levels of IL-3 for several months following transplantation in immunodeficient mice (Nolta *et al.*, 1994). Most recently, it has been shown that transiently transfected canine BM stromal cells transiently expressed clotting factor IX or human growth hormone *in vitro* and after autologous reinfusion in recipient dogs (Hurwitz *et al.*, 1997). However, in some studies, transplanted stromal cells did not engraft nor participate in the reestablishment of the BM microenvironment. The exact reasons for these differences are not fully understood but may in some cases reflect differences in cell culture methods (Keating *et al.*, 1982).

The persistence and FVIII expression of engineered BM stromal cells is presently under evaluation in animal models such as immunodeficient mice (Hoeben *et al.*, 1993; Nolta *et al.*, 1994), FVIII knockout mice (Bi *et al.*, 1995), and hemophiliac dogs (Giles *et al.*, 1982; Connelly *et al.*, 1996).

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NOTE ADDED IN PROOF

The highest FVIII production obtained to date in human BM stroma transduced with PG13-F8 under optimized conditions corresponded to 1500 ng/10^6 cells per 24 hr.

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